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Computational Modeling of Serum-Binding Proteins and Clearance in Extrapolations Across Life Stages and Species for Endocrine Active Compounds

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One measure of the potency of compounds that lead to the effects through ligand-dependent gene transcription is the relative affinity for the critical receptor. Endocrine active compounds that are presumed to act principally through binding to the estrogen receptor (e.g., estradiol, genistein, bisphenol A, and octylphenol) comprise one class of such compounds. For making simple comparisons, receptor-binding affinity has been equated to *in vivo* potency, which consequently defines the dose-response characteristics for the compound. Direct extrapolation of *in vitro* estimated affinities to the corresponding *in vivo* system and to specific species or life stages (e.g., neonatal, pregnancy) can be misleading. Accurate comparison of the potency of endocrine active compounds requires characterization of biochemical and pharmacokinetic factors that affect their free concentration. Quantitative *in vitro* and *in vivo* models were developed for integrating pharmacokinetics factors (e.g., serum protein and receptor-binding affinities, clearance) that affect potency. Data for parameterizing these models for several estrogenic compounds were evaluated and the models exercised. While simulations of adult human or rat sera were generally successful, difficulties in describing early life stages were identified. Exogenous compounds were predicted to be largely ineffective at competing estradiol off serum-binding proteins, suggesting this was unlikely to be physiologically significant. Discrepancies were identified between relative potencies based upon modeling *in vitro* receptor-binding activity versus *in vivo* activity in the presence of clearance and serum-binding proteins. The examples illustrate the utility of this approach for integrating available experimental data from *in vitro* and *in vivo* studies to estimate the relative potency of these compounds.

KEY WORDS: Bisphenol A; endocrine disruption; estradiol; genistein; octylphenol; PBPK model; risk assessment; serum protein binding; SHBG

1. INTRODUCTION

Endocrine active compounds are a large, structurally diverse group of compounds that interact with one or more components of the endocrine system, producing changes in hormone-regulated biological functions. The potential for toxicity is dependent upon the dose of the compound, timing and duration of exposure, and potency,[1] as well as other pharmacokinetic and pharmacodynamic factors.[2] Considerable interest and resources are being directed toward determining the dose-response behaviors of endocrine active compounds for disrupting endocrine-mediated functions. One major focus has been estimating relative potencies by determining relative-binding

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affinities for the estrogen receptor (ER) subtypes, ERα and ERβ, and the relative transcriptional activity in *in vitro* systems.[3–10] Methods to predict receptor-binding affinity from quantitative structure activity relationships are also under development.[11,12] Measures of *in vitro* binding activity may be useful, cost-effective screening tools as surrogates of *in vivo* potency. However, the diversity of mechanisms of action for endocrine active compounds, the complexity of the endocrine system, and the importance of pharmacokinetics, including serum-binding proteins and clearance, as modifiers of biological potency, indicate a broader approach would be of valuable.[13–18] In addition, identification of factors involved in pharmacodynamic specificity of endocrine active compounds, such as coactivators and corepressors responsible for some compounds acting as agonists in one tissue and antagonists in other tissues, have demonstrated the importance of characterizing more than just receptor binding in the processes leading to response.[19]

Four pharmacokinetic factors that can affect the availability of endocrine active compounds to intracellular receptors and thus can influence their apparent potency are plasma flow rate, binding to serum-binding proteins, factors altering cellular influx or efflux including altered membrane permeability, and metabolism.[20] Other factors that may be important *in vivo* are levels of endogenous estradiol (E2)[14] and feedback control of serum E2 levels.[16] Standard *in vitro* assays cannot adequately account for all these factors, but measurement and control of those that are relevant *in vitro* (i.e., serum protein binding, metabolism, and cell uptake) can facilitate extrapolation to the *in vivo* setting.

A central problem in the field of endocrinology has been the determination of whether the plasma concentration of free (nonprotein bound) hormone, or some combination of free and bound hormone, controls intracellular hormone concentrations.[20] Both experimental[21–24] and theoretical analyses[20] support the conclusion that there are conditions under which both free hormone and bound hormone contribute to intracellular concentrations of hormone.

For tissues where the test compound is not metabolized or metabolism is sufficiently slow to be rate limiting, modeling by Mendel indicates that intracellular concentrations at steady state are controlled by the free concentration of the compound in serum.[20] When metabolism is absent from *in vitro* systems, the concentrations and binding characteristics of serum proteins similarly are critical determinants of intracellular concentrations, receptor binding, and biological activity. By contrast, when metabolism is relatively rapid or blood flow or dissociation from binding proteins is limiting, the intracellular concentrations are not directly related to free concentrations in serum. Hence, the clinical focus on "bioavailable" estrogen (in this context used to mean free plus "weakly bound" estrogen, largely albumin bound) in contrast to free estrogen.[25] Relative receptor-binding affinity estimates generated in the presence of rodent serum also may not be successfully extrapolated to humans because of species-specific differences in the content and binding characteristics of serum proteins.[14] Extrapolation across life stages should similarly address differences in serum-binding proteins, such as occur during pregnancy or early in life.

Computational modeling was used to characterize the influence of these different pharmacokinetic factors on the biological activity of endocrine active compounds. The *in vitro* model described effects of measuring the apparent binding affinities of E2 and other endocrine active compounds in the presence of male and female human serum, adult rat serum, perinatal rat serum, human cord blood, or under serum-free conditions. In addition to E2, the endogenous ER ligand to which the potency of estrogenic agonists is typically compared, genistein, bisphenol A, and octylphenol, were analyzed. The *in vitro* model provided a framework for integrating compound-specific tissue or cell partitioning (distribution) and protein- and receptor-binding data to estimate comparative biological activity, but it did not address metabolism or other forms of clearance. To describe the impact of differential clearance on potency, the *in vitro* model was extended to an *in vivo* two-compartment model.

2. METHODS

2.1. Modeling Approach

The equations for the *in vitro* model and parameter values for E2 were validated by simulating the free fraction of E2 in adult human and rat sera.[14,26,27] Subsequent modeling of *in vitro* conditions characterized the influence of serum-binding proteins on the free concentration and receptor-binding activity of selected endocrine active compounds (i.e., E2, genistein, bisphenol A, and octylphenol) in an experimental design that used MCF-7 cells. These results further validated the E2 parameterizations, while for genistein, bisphenol A, and octylphenol the assays were used to estimate parameter values.

Extending simulations to additional life stages depends on knowing the changes in concentrations of serum-binding proteins with life stage. Since αFP and SHBG concentrations vary with life stage in rodents and humans, respectively, these species must be addressed separately.[17,28,29] Albumin concentrations also change during pregnancy[30,31] and *in utero* development.[28,32] Available data were used to evaluate the success of the model for describing life stages other than adulthood.

Finally, a limited model describing *in vivo* clearance of endocrine active compounds was developed. This model permitted comparisons of the impact of serum-binding proteins and clearance on the apparent receptor-binding activity of endocrine active compounds *in vitro* versus *in vivo*.

2.2. *In Vitro* Ligand-Binding Model Structure

A two-compartment model representing media with binding proteins and cells expressing receptor protein was prepared using ACSL (v11.8.4, AEGIS Technologies, Huntsville, AL) (Fig. 1). This *in vitro* structure also can be parameterized to simulate serum-binding experiments (i.e., no receptor containing cells). It describes the equilibrium binding of two ligands and two binding proteins, the equilibration of the free ligands across a tissue barrier as a function of tissue:media partition coefficients, and subsequent equilibrium binding to a cellular receptor. The binding protein description consisted of a series of simultaneous mass balance equations (see the Appendix) that solve for free and bound concentrations of all binding partners. This structure was used in both the *in vitro* and *in vivo* models.

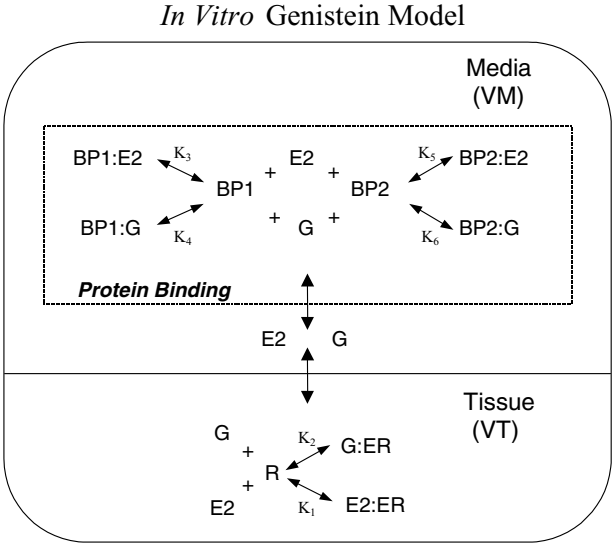

Fig. 1. Diagram of the *in vitro* model detailing estradiol (E2) and genistein (G) binding to binding proteins (BP1 and BP2) and the estrogen receptor (ER) in the media and tissue compartments, respectively. VM = volume of media compartment. VT = volume of tissue compartment.

2.3. Parameterization of the *In Vitro* Model and Assessment of Available Data

Tables I and II present the values used for the dissociation constants, protein concentrations, tissue partition coefficients, and volumes. Parameters were obtained to the extent possible from the literature, while others had to be estimated from the experimental studies using the model.

2.4. Dissociation Constants

2.4.1. Estradiol

The K_D used for E2:ER binding was 0.2 nM.[15,33] This value is plausible based upon a review of published values, which are highly variable.[34] Estradiol has similar affinities for ERβ and ERα.[35,36]

The K_Ds for E2 binding to albumin, SHBG, and αFP have been reported. Dissociation constants for E2 with albumin have been reported to range from 17 to 50 μM;[37] 17 μM was selected because it had been used in previous modeling describing steroid hormones in human serum.[38] The human albumin binding affinity was also assumed to apply to rat albumin because no measurements were located in the published literature. A dissociation constant of 1.54 nM for E2 binding to SHBG was used based upon previous modeling[38] and consistent with experimental studies.[37,39] The K_D of E2 for rat αFP has been reported between 15 and 18 nM[40–42] except for one estimate of 3.5 nM;[43] a value of 16 nM was used here. Human αFP does not have measurable binding affinity for E2.[44,45] While αFP needs to be considered for describing dosimetry of ER agonists in rats, it does not need to be considered for the human if, as assumed here, other endocrine active compounds also do not bind to it.

2.4.2. Genistein

Binding affinities of genistein for ER and SHBG were available in the literature. The K_Ds for albumin and αFP have not been reported, so these parameters

Table I. Parameter Values for *In Vitro* Model

Parameters	Units	Rat	Human	Citation
Compartment volumes				
Tissue	L	0.001	0.001	Assumed (see text)
Media	L	1	1	Assumed
Tissue:plasma (media) partition coefficients				
Estradiol		1.0	1.0	Assumed (see text)
Genistein		1.0	1.0	Assumed
Bisphenol A		1.0	1.0	Assumed
Octylphenol		1.0	1.0	Assumed
Binding proteins-serum concentration				
Albumin concentration				
Adult	nM	410×10^3	500×10^3	[30,55]
Pregnant	nM	410×10^3	400×10^3	[30,56]
Cord blood	nM	NA	52.7×10^3	[58]
SHBG concentration				
Male adult	nM	NA	20	[30]
Female adult	nM	NA	40	[30]
Pregnant female	nM	NA	400	[30]
Cord blood	nM	NA	44	[25]
α-fetoprotein concentration				
Adult	nM	0.25	NA	[40]
Estrogen receptor α	nM	59.2	59.2	[61]

Note: Unless noted, values are for adult male rats or humans. NA—not applicable.

were estimated by fitting experimental data with the *in vitro* model.

Genistein binds with greater affinity to ERβ than ERα, but the parameterization was for ERα, consistent with the receptor content in MCF-7 cell studies that were evaluated.[46] Relative-binding affinities of genistein for ERα compared to E2 were reported as 1.6% and 5%,[35,36] so a genistein K_D for ERα of 10 nM was used, 2% of the E2 affinity. The relative-binding affinity of genistein for SHBG was less than 1% compared to E2 in two studies.[39,47] A dissociation constant of 1,590 nM (corresponding to a relative-binding affinity of 0.1%) was used based upon the affinity constant in Dechaud *et al*.[39]

Table II. Dissociation Constants for Protein-Endocrine Active Compound Binding

Parameters	Units	Rat	Human	Citation
Estrogen receptor				
Estrogen	nM	0.2	0.2	See text
Genistein	nM	[a]	10	[36,52]
Bisphenol A	nM	[a]	400	[52,53]
Octylphenol	nM	[a]	285	[15,52]
Albumin				
Estrogen	nM	[a]	17,000	[38]
Genistein	nM	[a]	200,000	Fitted
Bisphenol A	nM	[a]	41,000	Fitted
Octylphenol	nM	[a]	500	Fitted
SHBG				
Estrogen	nM	NA	1.54	[38]
Genistein	nM	NA	1,600	[39]
Bisphenol A	nM	NA	13,600	[39]
Octylphenol	nM	NA	2,000	[39]
α-fetoprotein				
Estrogen	nM	16	NA	[39,40]
Genistein	nM	13,000	NA	Fitted
Bisphenol A	nM	$\geq$13,000	NA	See text
Octylphenol	nM	$\geq$13,000	NA	See text

[a]Human values assumed for rat. NA—not applicable.

The *in vitro* model was used to infer the value of the genistein:albumin K_D from experiments. Competitive displacement by genistein of ER bound radiolabeled E2 was determined in cultured MCF-7 cells in the presence and absence of human male serum.[14] Using the parameter values in Tables I and II, we identified a value of the genistein:albumin K_D that fitted the approximately 11-fold difference between the free fractions of genistein and E2 (45.8% and 4.0%, respectively) in the presence of human male serum. The predicted value of the genistein:albumin K_D was 200 μM by comparison with the E2:albumin K_D used here of 17 μM.[38] An alternative derivation of the dissociation constant for genistein binding to albumin is to use the reported 45.8% free genistein directly.[14] A genistein:albumin K_D of 430 μM was required to predict this free concentration. Both estimates indicate that genistein binds albumin with 10–20-fold less

affinity than E2. These estimates of albumin-binding affinity are dependent upon the assumption of a single binding site for genistein on each albumin molecule and the assumed concentration of albumin in the male serum.

There is a published value for the binding affinity of genistein for SHBG,[39] as well as reports that too little competition was observed to estimate an inhibition constant.[48–50] Hodgert-Jury and co-workers reported 57.8% ± 24.5% of protein-bound E2 was displaced by 200 μM genistein in undiluted human pregnancy serum. A K_D of 4.0 μM was estimated using the model to compute 58% of the E2 bound to SHBG (conditions: 370 nM SHBG reported, 40 nM E2 reported, 400 μM albumin for pregnancy serum). This K_D estimate is only 2.5-fold different from the value of 1.6 μM based upon Dechaud *et al.*[39]

Binding of genistein to αFP has been described,[48,51] but a value for the genistein:αFP K_D has not been determined by standard methods. Baker and co-workers[51] used a competitive ligand-binding assay to measure inhibition of [^{3}H]-estrone binding to αFP by a single concentration of genistein. The concentration of rat αFP used in the assay was low enough to allow valid measurements of inhibition (i.e., below their measured estrone:αFP K_D of 3.3 nM). Under the experimental conditions (αFP: 0.9 nM, estrone: 3.0 nM, and genistein: 3000 nM), 10% inhibition of [^{3}H)-estrone binding to αFP was reported. The genistein:αFP K_D estimated by fitting this data with the model is 13 μM, similar to the approximate value of 5 μM reported without explicit derivation by Baker and co-workers.

2.4.3. Bisphenol A

Like genistein, bisphenol A appears to have a greater affinity for ERβ than for ERα (Kuiper, 1998 #4371; Matthews, 2001 #5457; Kuiper, 1997 #2234). Estimates of its relative affinity for ERα compared to E2 range from 0.006% to 0.05%.[14,52,53] A K_D of 400 nM (representing a relative affinity of 0.05% compared to the K_D of 0.2 nM for E2) was used here.

Binding of bisphenol A to SHBG has been reported.[39,48,50] The dissociation constant of 13.6 μM was calculated from the association constants reported by Dechaud *et al.*[39] Results in the other two papers indicate similar or less tight binding.

Binding of bisphenol A in serum has been measured and largely reflects binding to albumin.[54] The number of binding sites and dissociation constant based upon saturation equilibrium-binding experiments were 2,000 and 100 μM, suggesting multiple binding sites on albumin. Predicting the 7.8% free bisphenol A reported by Nagel *et al.* required a K_D of 41 μM in the model, assuming one binding site per albumin.[14]

Limited data are available for estimating a binding affinity of bisphenol A for αFP. Milligan *et al.* reported no displacement by bisphenol A of radiolabeled E2 from αFP from rat amniotic fluid. They reported a similar result for genistein, though estimates based upon Baker *et al.*[51] give approximately a 1,000-fold lower affinity for genistein compared to E2 (see above). Therefore, the maximum affinity of bisphenol A for αFP would be that determined for genistein (13 μM), although it may actually bind even less well.

2.4.4. Octylphenol

4-octylphenol has a binding affinity of 0.02–0.07% of the affinity of E2 for ERα and ERβ, respectively, measured using *in vitro* assays.[15,52] Based upon this, the parameterization for octylphenol used a dissociation constant of 285 nM representing 0.07%. 4-*tert*-octylphenol was reported to have a similar relative-binding affinity of 0.01 and 0.03% for ERα and ERβ, respectively.[52] Binding of octylphenol to SHBG has been reported.[39,48,50] The dissociation constant used (2 μM) was calculated from the association constant reported by Dechaud *et al.*[39] The results in the other two papers indicate similar or less tight binding. To predict the reported 0.3% free octylphenol in human male serum,[14,15] a value of 500 nM (0.5 μM) was obtained for the dissociation constant for octylphenol for albumin. Based upon limited data, as described above for bisphenol A, a value of 13 μM was used for the K_D for αFP, though the affinity actually may be even lower.

2.5. Protein Concentrations

2.5.1. General

Concentrations of SHBG, albumin, and αFP in rat and human sera were obtained from the literature. For rat serum, the concentrations of αFP and albumin were set to the levels present in adult rats[40,55,56] or developing rats[28,29] as appropriate for the simulation. Rat albumin concentrations do not decrease during pregnancy in contrast to humans.[56] For human serum, the albumin and SHBG concentrations were those of an adult male and female, pregnant female, and cord blood, depending on the analysis.[38,57,58] The

albumin concentration used by Dunn and co-workers is at the low end of the range reported for adult human albumin concentrations (507–725 μM).[32] Concentrations of serum proteins were assumed constant during the phases of cycling in nonpregnant females.

2.5.2. Perinatal Rats

For gestational Day 19, Dziegielewska and co-workers report fetal plasma concentrations of 583 ± 62 mg/100 ml for albumin (88.3 μM) and 441 ± 22 mg/100 ml for αFP (61.3 μM).[28] This αFP concentration is similar to the 4.91 mg/ml reported by Lai and co-workers.[29] In newborns and on Day 2 αFP was reported to be 322 ± 15 and 208 ± 19 mg/100ml (44.7 and 28.9 μM), while albumin concentrations were 1,485 ± 117 and 2,250 ± 151 mg/100 ml (225 and 341 μM).[28]

2.5.3. Human Fetus

Human data are available for estrogens and binding proteins present in cord (generally venous) and maternal blood.[25,59] No human data directly comparable to rat data were identified, i.e., free and bound concentrations in fetal and early postnatal serum, though total fetal serum hormone early in pregnancy have been reported.[60] Shibata *et al.*[25] report cord blood at term to contain 57.7 nM total E2, 53.1 nM "bioavailable" E2 ("bioavailable" refers here to free and albumin-bound E2 combined), 144 nM estrone, and 44.4 nM SHBG; values from Adlercreutz and Luukkainen are approximately half that for total E2 and estrone.[59] It is notable that human estrogen levels are far higher than those reported for rodents.

2.6. Tissue Partitioning and Estrogen Receptor Content

The tissue:media partition coefficient for E2 was fixed at 1.0, though as discussed for efforts to model E2 pharmacokinetics, the correct value likely would be higher when serum and tissue-binding proteins are explicitly described.[34] Measured tissue:blood partition coefficients have been reported for bisphenol A.[54] Partition coefficients of 1.0 were assumed for the other compounds. The ER content of the cells was set to 59 nM, the content reported in rat uterine tissue;[61] no data on MCF-7 cell concentrations were located. The cell volume was assumed to be 0.1% of the media volume, consistent with the relatively small volume of a single confluent layer of test cells as would be used for many expression assays.

2.7. *In Vivo* Model Structure

A simple *in vivo* model was constructed to represent clearance processes affecting receptor-binding activity for comparison with the simulated *in vitro* results. The model has plasma and response tissue compartments. The plasma compartment treats intravenous (i.v.) infusion delivery and first-order elimination of administered compounds, in addition to binding to serum proteins. The response tissue compartment is representative of uterine tissue, with corresponding volume, blood flow, and ER content (Tables I–III).

2.8. Parameterization of the *In Vivo* Model

The model is illustrated in Fig. 2 and the physiological parameters used (i.e., plasma and response tissue volumes and blood flows) are found in Table III. The response tissue:blood partition coefficients for all compound were assumed equal to that for E2 (Table III). Concentrations of serum proteins and affinity constants were simulation specific and are listed in Tables I and II.

The first-order elimination rate constants for E2, genistein, and bisphenol A were estimated by fitting blood or plasma concentration versus time data to a classical two-compartment pharmacokinetic model coded in ACSL. The equations for the two compartments were $dA_1/dt = -k_{12}A_1 + k_{21}A_2 - k_{10}A_1$ and $dA_2/dt = -k_{21}A_2 + k_{12}A_1$, where A_i is the amount in Compartments 1 and 2, k_{12} is the transfer rate from Compartment 1 to 2, k_{21} is the transfer rate from Compartment 2 to 1, and k_{10} is the rate of first-order clearance. Visually good fits were obtained with data at a single dose for each of the three compounds (simulations not shown). A first-order elimination rate constant of 0.1 h^{-1} was estimated by fitting the plasma pharmacokinetics of E2 in male rats following i.v. administration of 20 μg of E2 reported by Bawaarshi-Hassar and co-workers.[62] The i.v. pharmacokinetics of bisphenol A (10 mg/kg) in ovariectomized female DA/Han rats[63] were best fit by a first-order elimination rate constant of 0.056 h^{-1}. Unpublished data on the i.v. blood pharmacokinetics of genistein in male Wistar rats (Coldham, N., personal communication, 2002) were used to estimate a first-order elimination rate constant of 0.05 (female) to 0.09 (male) h^{-1} for genistein; the female value was used in subsequent analyses. These rate constants were used directly in the *in vivo* model as estimates of the rates of clearance of these compounds assuming they represent

Table III. Parameter Values for the *In Vivo* Model

Variable Name	Units	Value	Citation
Body weight	kg	0.350	Assumed adult rat
Compartment volumes			
Response tissue	L	0.002	[78]
Plasma	L	0.04	[79]
Flow rates			
Cardiac output (plasma)	L/h/kg$^{(75)}$	7.7	[34]
Response tissue	(% CO)	0.25	[34]
Partition coefficients RT[a]			
Estradiol		1.0	[34]
Genistein octylphenol, bisphenol A		1.0	Assumed equal to estradiol
Clearance rates (h^{-1})			
Estradiol		0.1	Fitted (see methods)
Genistein		0.05	Fitted (see methods)
Bisphenol A		0.056	Fitted (see methods)

[a]Response tissue, parameterized as uterine tissue.

clearance below saturation. Although, the pharmacokinetics of these compounds are more complicated than can be fully represented by first-order elimination rate constants (e.g., enterohepatic recirculation for genistein), their use as relative measures of clearance was reasonable for the analyses presented here.

3. RESULTS

3.1. Evaluation of Model Structure and Parameterization Using Data for E2 and Adult Human and Rat Serum

To test the ability of the *in vitro* model to replicate experimental results, determinations of E2 binding by rat and human serum and the influence of serum on the free fraction of E2 available for receptor occupancy were simulated.[14,26,27]

Binding of E2 to serum proteins was measured in sera from adult (age unspecified) male and female humans and female rats in diestrus and proestrus using ultrafiltration dialysis.[14,26,27] These serum-binding studies were simulated using a media only parameterization that eliminates binding to tissue ER (affinity constant for E2 binding to the ER set high: $k_1 = 1{,}000{,}000$). Addition of 4.2 nM E2 to human adult male serum resulted in an experimentally determined 2.36% ± 0.08% free E2.[14] The model predicts 2.35% free at this concentration using parameter values in Tables I and II. Similarly, the predicted free E2 was 2.34% and 2.36% in the presence of human male serum and 3 or 6 nM E2, while values of 2.42% ±

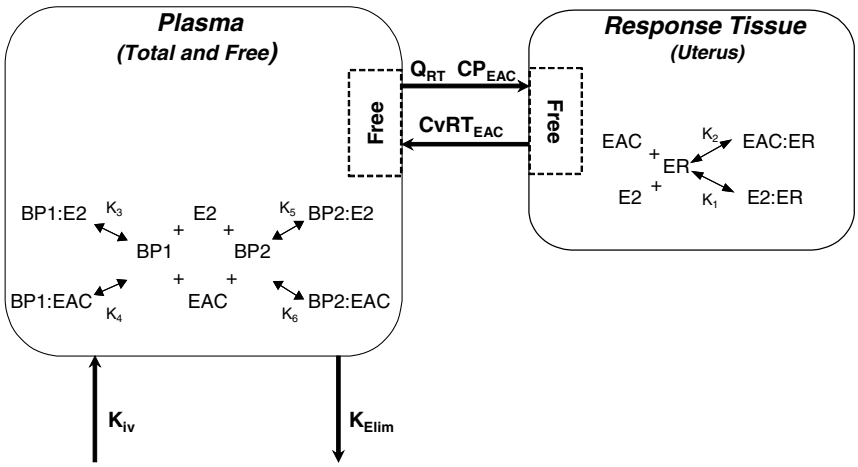

Fig. 2. Diagram of the *in vivo* model detailing estradiol (E2) and endocrine active compound (EAC) binding to binding proteins (BP1 and BP2) and the estrogen receptor (ER) in the media and response tissue compartments, respectively. The response tissue approximates a uterine compartment. Free endocrine active compound (CP_{EAC}, $CvRT_{EAC}$) is exchanged between the plasma and response tissue compartments through blood (plasma) flow (Qrt). Estradiol is similarly described. Elimination is described as first order.

Table IV. Experimentally Measured and Modeled Binding of E2 in Sera of Rats of Different Ages and Hormonal Status

			Experimental Data			Model Results		
Rat Serum	Albumin (nM)	AFP (nM)	Total E2[a] (pg/ml)	Total E2[a] (nM)	Free E2 (nM)	Free E2 (%)	Free E2 (nM)	Free E2 (%)
Proestrus	410,000	0.25	59.7 ± 7.7	0.22	0.0083	3.9 ± 0.12	0.0087	4.0
Diestrus	410,000	0.25	13.7 ± 1.7	0.050	0.0019	4.03 ± 0.17	0.0020	4.0
gd19	88,300	61,300[b]	155 ± 4	0.57	0.0020	0.35 ± 0.01	0.00015	0.027
		4,460[c]					0.0020	0.35
pnd 1	225,000	44,700[b]	126 ± 6	0.463	0.0080	1.77 ± 0.20	0.00016	0.035
		700[c]					0.008	1.73
pnd 2	341,000	28,900[b]	5.6 ± 0.6	0.0206	0.00026	1.12 ± 0.09	0.000011	0.054
		1,000[c]					0.00025	1.2

[a]Montano *et al*. (1995).
[b]Measured values reported by Dziegielewska *et al*. (1981).
[c]Value required for model to model measured percent free E2.

0.10% and 2.58% were obtained experimentally.[27] For human female serum, assuming 40 nM SHBG, the model predicts 1.80% and 1.83% free at 3 and 6 nM E2, while using 61 nM SHBG (as measured in Hodgert Jury *et al*.[50]), the free E2 was 1.45% and 1.47% at 3 and 6 nM E2. These values compare reasonably well with the reported values, which range from 1.51% to 1.58%.[27] The menstrual-cycle stage of these women was unreported.

Binding of E2 in female rat serum was estimated using radioimmunoassay to determine the total E2 concentration and centrifugal ultrafiltration dialysis to determine the free concentration.[26] Using the reported total E2 concentration for proestrus and diestrus rats and parameter values in Tables I and II, the model successfully describes the experimental data (see Table IV).

The ER occupancy in cultured MCF-7 cells was measured in the presence and absence of human male serum to determine the free fraction of E2.[14] The free fraction of E2 was calculated using the media concentrations of E2 required for 50% receptor occupancy in a competition assay with radiolabeled E2; the concentration in serum-free medium was divided by the apparent K_D in the presence of adult male serum. Using parameter values in Tables I and II, the model was used to run a dose-response curve for E2 binding to ER (Fig. 3). The resulting free fraction of E2 in human male serum is 2.4%, closer to the value calculated by ultrafiltration dialysis (2.4%) than to the values of 3.46 ± 0.20 and 3.97% ± 0.18% reported using the MCF-7 saturation and competition assays, respectively. Nagel and co-workers[14] report an average K_D for E2 binding to ER in serum-free medium of 0.095 ± 0.035 nM and an average apparent K_D in the presence of male serum of 2.62 ± 0.81 nM. Using 0.095 nM for the K_D, the dissociation constant for E2 binding to albumin is estimated to be 35 μM, about double that in Tables I and II, to predict the apparent K_D of 2.6 nM and the free fraction as 3.6%. An E2:albumin K_D of 30 μM was reported by Moll *et al*.[37]

Overall, these simulations are verification that the model correctly treats binding of a ligand with one or more plasma-binding proteins and can be used for simulating E2-free concentrations and receptor binding under experimental conditions with or without sera from adult rats or humans.

3.2. Sex and Life Stage in Predictions of E2-Free Fraction in Human Serum

The predicted free fraction of E2 in human male serum is 2.4% (Fig. 4) until supraphysiological concentrations when small increases occur. For E2 concentrations near those found in nonpregnant women (0.3–0.7 nM for follicular and luteal phases, respectively),[30,38,64] the predicted free fraction is 1.8% in nonpregnant human female serum, again rising only modestly at very high E2 concentrations as SHBG beings to saturate (Figs. 4 and 5A). The free fraction of E2 in human pregnancy serum is more restricted due to high concentrations of circulating SHBG (400 nM).[38] Again, the free fraction is predicted to be relatively constant at 0.4% until concentrations higher than those observed *in vivo* for E2 (55 nM, 3rd trimester) (Fig. 6).

Modeling of the free and bound E2 in human cord blood as measured by Shibata *et al.*[25] was

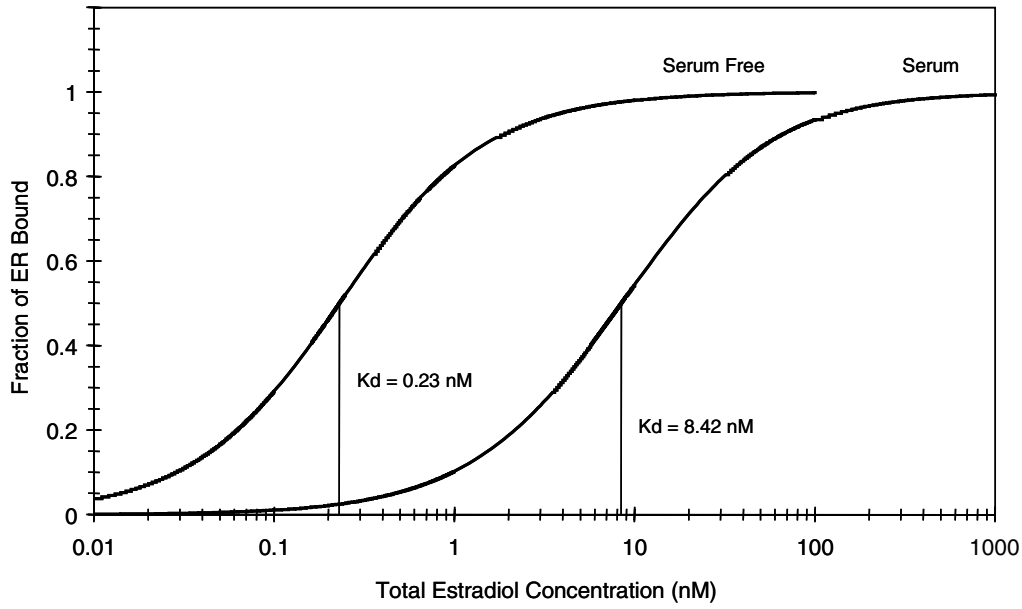

Fig. 3. The effect of human male serum on the modeled cellular estrogen receptor binding as a function of E2 concentration. Serum contained male specific concentrations of SHBG and albumin.

generally unsuccessful whether the analysis was done just for E2 or both E2 and estrone. Using the measured concentrations of E2 (57.7 nM or 1570.7 ng/dl) and SHBG (44.4 nM) and assuming 527 μM albumin in cord blood,[58] the model predicts 1.2 nM free E2 (2% free), 37.1 nM albumin-bound E2, and 19.4 nM SHBG-bound E2. This represents much less "bioavailable" (this clinical endocrinology usage meaning free plus albumin-bound: 38.3 nM predicted vs. 53.1 measured) than the measured data indicate.

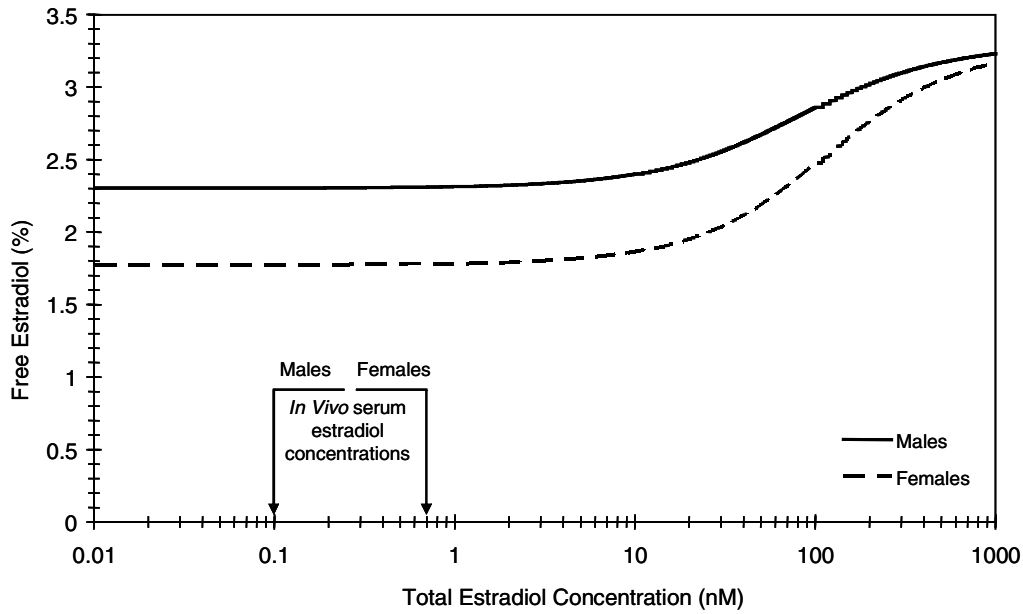

Fig. 4. The effect of increasing E2 concentration on the predicted free fraction of E2 in human male and nonpregnant female serum. The levels of endogenous E2 are noted.

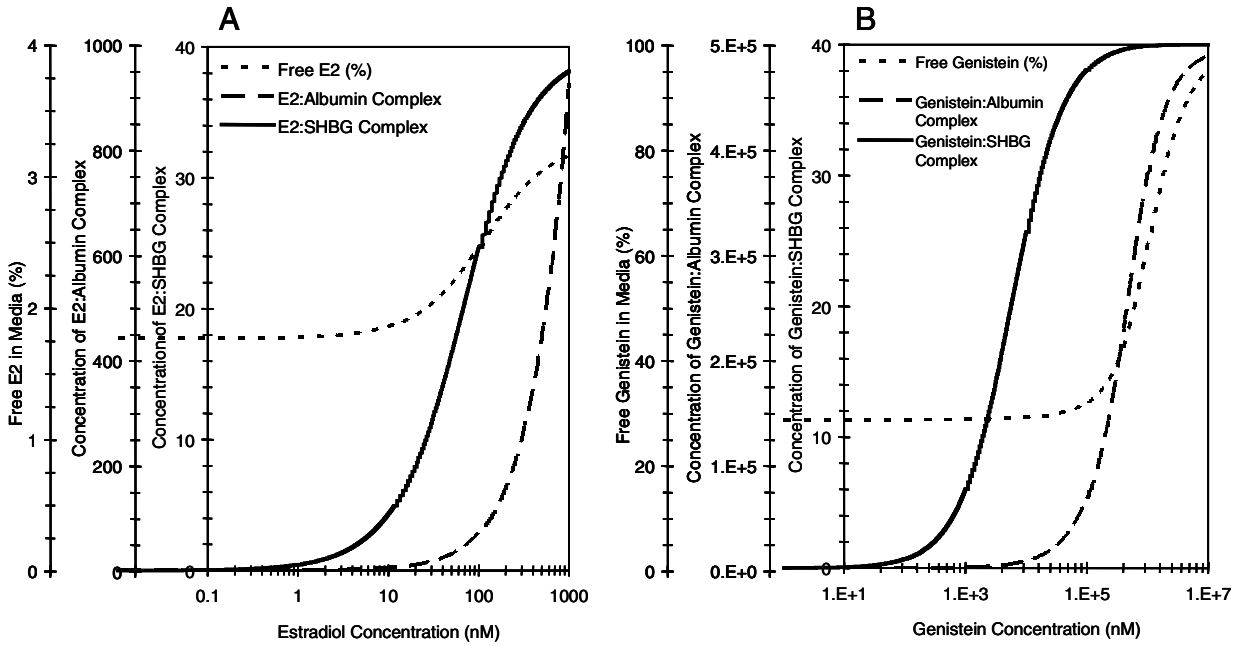

Fig. 5. The relationship between E2 and genistein-free fraction and binding to SHBG and albumin in female human serum.

Modeling of both E2 and estrone reduces the SHBG-bound E2 due to competition by estrone and increases the albumin bound E2 (free E2: 1.4 nM or 2.4% free, albumin-bound E2: 42.1 nM, SHBG-bound E2 14.2 nM, free estrone 5.9 nM, albumin-bound estrone 124 nM, SHBG-bound estrone 14.1 nM using an estrone:albumin $K_D = 25\ \mu M$ and estrone:SHBG $K_D = 6.7$ nM, both from Dunn *et al.*[30]). The sum of the free and albumin-bound E2 is now estimated at 43.5 nM, versus the measured 53.1 nM. These results

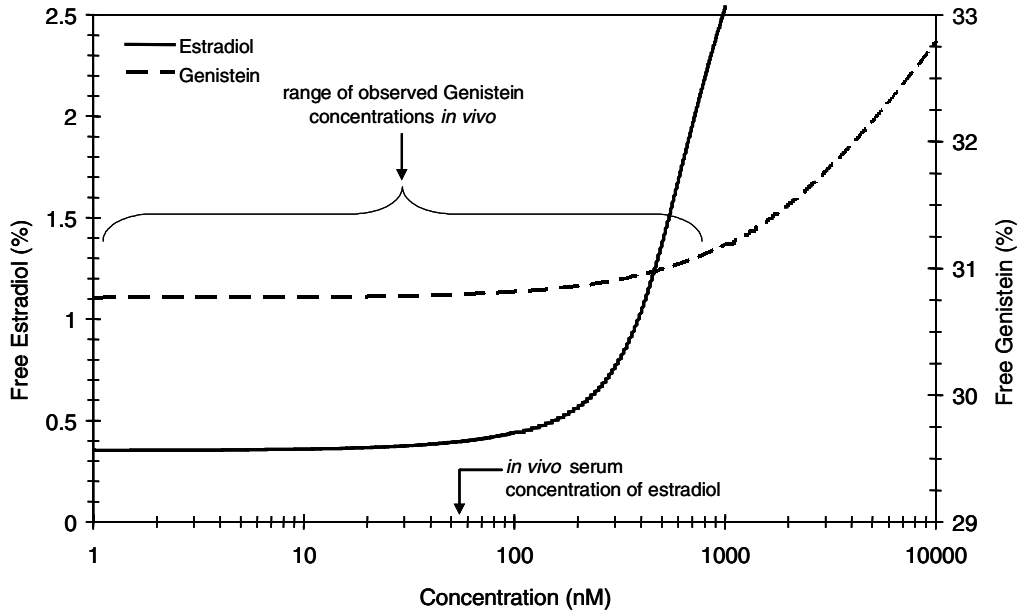

Fig. 6. The effect of concentration on the modeled free fraction of E2 and genistein in human pregnancy serum. Serum was free of endogenous E2.

with cord blood contrast with the ability of the model to replicate data for measured free E2 in adult human serum.

3.3. Sex and Life Stage in Predictions of E2 Free Fraction in Rodent Serum

Adult Rats: Prediction of the free fraction of E2 in adult rats was undertaken as part of the evaluation of the model, described above. Predicted free fractions of E2 were in agreement with measured values of ~4.0% (Table IV).

Perinatal Rats: Binding of E2 in rat serum during gestation and shortly after birth was simulated for comparison with the data of Montano *et al.*[26] The authors report the concentrations of total E2 and free E2, based upon measurement of the free fraction, in fetal serum on gestational Day 19 and in neonatal serum 4 and 48 h after birth (Table IV).[26]

The model results show virtually all the E2 bound to αFP (0.569 nM), very low binding to albumin (0.0008 nM), and 0.00015 nM free E2 or approximately 0.027% free on gestational Day 19. This is approximately 10-fold less free E2 than the measured 0.35%. To achieve the reported free E2, the concentration of αFP must be reduced to 4.46 μM from 61.3 μM. Given the very low concentration of E2 (0.57 nM) and the high concentration of αFP, the adjusted αFP concentration represents the apparent free αFP available for binding with the E2. The predicted E2-free fractions of 0.035% and 0.054% at 4 and 48 h after birth were much lower than the measured values of 1.8% and 1.1%. To predict the measured free fractions, the αFP concentration must be reduced to 0.7 and 1.0 μM from 44.7 and 28.9 μM. These adjustments in αFP concentrations are consistent with the discussion by Montano and co-authors that the differences in free fraction they observe are not consistent with changes in αFP concentration.

3.4. Comparisons of Modeled Genistein, Bisphenol A, and Octylphenol-Free Fractions and Receptor-Binding Activity in Human Serum

Simulations with genistein, bisphenol A, and octylphenol were conducted for *in vitro* receptor occupancy essays in the absence and presence of human serum from adult male, adult nonpregnant, and pregnant females (Table V). In contrast to the large variations in E2-free fraction with life stage and sex, the free fraction of genistein, bisphenol, and octylphenol (predicted at 50% receptor occupancy) change by less than 25% in the presence of adult male, female, and pregnancy serum. The basis for this difference between these xenoestrogens and the endogenous hormone is the relative importance of albumin binding and the insignificance of SHBG binding at the concentrations required for 50% occupancy of ERα. This is illustrated for genistein in Figs. 5 and 6. As is observable in Fig. 5, the free fraction of E2 begins to increase as SHBG-binding saturates (Fig. 5A), while for genistein, saturation of SHBG-binding sites has no impact and the free fraction begins to rise only as albumin-binding sites are increasingly occupied.

Table V. Modeled EC_{50}s and Relative Potencies for *In Vitro* Assays of Receptor Binding with Human Serum

	No Serum		Adult Male Human Serum		Adult Female Human Serum		Pregnant Human Serum	
Assay	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)
Estradiol	0.2	100	8.4	2.4	11	1.9	51	0.4
Genistein	10	100	35	28	35	28	32	31
Bisphenol A	400	100	5231	7.7	5231	7.7	4271	9.4
Octylphenol	285	100	181,594	0.16	181,594	0.16	144,861	0.20

	Potency Relative to E2[a]			
	No Serum	Adult Male Human Serum	Adult Female Human Serum	Pregnant Human Serum
Estradiol	1	1	1	1
Genistein	0.02	0.24	0.24	1.59
Bisphenol A	0.0005	0.0016	0.0021	0.12
Octylphenol	0.0007	0.000046	0.000046	0.00035

[a] EC_{50}E2/EC_{50}EAC.

The simulated receptor binding EC_{50}'s for genistein and bisphenol A are ~4–13 times higher in the presence of human serum compared with serum-free assays. Higher affinity binding of octylphenol to albumin restricts its free fraction. Simulations indicate that octylphenol receptor binding EC_{50}s are 600 times higher in the presence of serum than in serum-free assays. The impact of serum binding on *in vitro* relative activity of the compounds as compared to E2 can be estimated by comparing the ratios of the simulated EC_{50}s for receptor binding (Table V). These simulations show that relative to E2, the *in vitro* activities of genistein and bisphenol A increase in the presence of serum-binding proteins, while that of octylphenol decreases. The predicted relative activity of genistein for ER binding exceeds (1.6 times higher) that of E2 in the presence of pregnancy serum, in stark contrast to prediction for serum-free conditions, which indicate that the receptor-binding activity of genistein is 50 times lower than E2.

Finally, simulations were run of free E2 concentration in the presence of increasing genistein, bisphenol A, and octylphenol under physiological serum conditions for male, female, and pregnancy sera (Figs. 7 and 8). These simulations show no change in E2-free fractions till very high competitor concentrations are observed. Similarly, predicted ER occupancy is essentially unaffected till very high concentrations, except in the case of genistein, which substantially increases occupancy in the presence of male and nonpregnant female E2 concentrations and serum-binding proteins due to its own relatively high affinity for the ER.

3.5. Comparisons of Modeled Genistein, Bisphenol A, and Octylphenol-Free Fractions and Receptor-Binding Activity in Rat Serum

In vitro receptor-binding assays in the presence of rat serum from different life stages were modeled for genistein, bisphenol A, and octylphenol to identify the concentration required to give 50% receptor binding and the free fraction of these compounds at that EC_{50} (Table VI). Simulation of the rodent fetal and early postnatal serum binding of genistein, bisphenol A, and octylphenol was hampered by two issues: poorly determined binding affinities for αFP and uncertainty concerning the concentration of αFP available for binding. Modeling was undertaken to estimate the expected behavior of these compounds within the limitations of the available data. Overall, the free fractions of bisphenol A and genistein are much higher than E2 (Table VI). With the exception of GD19, octylphenol is more highly bound to albumin and has free fractions lower than E2 across the whole perinatal and adult period. In the absence of metabolism in the *in vitro* model, these changes in free fraction result in corresponding changes in free concentration and EC_{50}s (Table VI). The net impact of changes in serum binding of these endocrine active compounds relative to E2 *in vitro* on relative potency based upon ER binding can be estimated by comparing the ratios of the simulated EC_{50}s for receptor binding (Table VI). Results in Table VI were predicted in the absence of endogenous E2, but the results vary by less than 5% in all the cases if the endogenous concentrations of E2 reported by Montano *et al.*[26] are incorporated (data not shown). These results, consistent with experimental measurements, show that binding to serum proteins reduces the free concentration and thus the effective concentration at the receptor, as well as altering the apparent relative potencies for *in vitro* receptor binding.

3.6. Impacts of Clearance *In Vivo* on Relative-Binding Activity

The rodent uterotrophic assay is a standard method for evaluating estrogenic potential *in vivo*. The *in vivo* rat model, composed of a plasma and uterine compartment, was used to simulate the combined impact of serum-protein binding and chemical-specific clearance on *in vivo* activity. The fraction of uterine ER bound, and the total and free concentrations of E2, genistein, and bisphenol A were simulated at steady state following equal i.v. infusion rates (0.005 nmol/h). In direct relation to their lower clearance, the steady-state plasma concentrations of genistein and bisphenol A were approximately two-fold higher than E2. The resulting free concentrations, or we presume here, biologically available concentrations of genistein and bisphenol A are 16 and 4 times higher than E2, respectively. These simulations were done without "endogenous" E2, as it would be virtually absent in the uterotrophic assay.

Model-estimated relative potencies based upon receptor binding *in vivo* can be defined as the ratio of the doses (dose rates) of E2 and the test compound required to produce 50% uterine ER occupancy (analogous to an ED_{50}). Higher dose rates for genistein (0.0155 nmol/h) and bisphenol A (2.5 nmol/h) and much higher plasma concentrations are necessary to achieve 50% occupancy of uterine ERs for these compounds. The resulting relative potencies

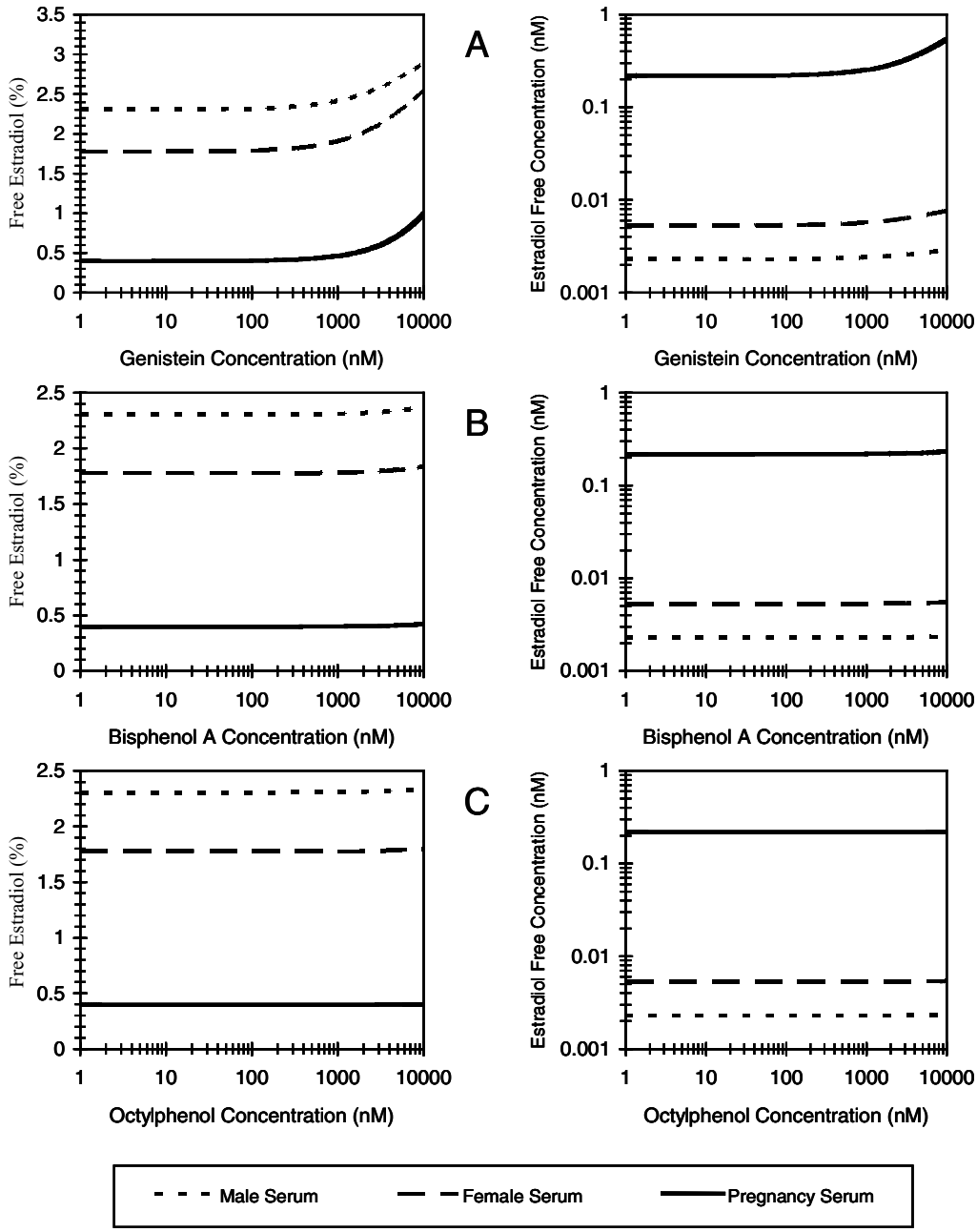

Fig. 7. The effect of co-exposure to (a) genistein, (b) bisphenol A, and (c) octylphenol on the free fraction and free concentration of E2 in human male, female, and pregnancy serum. Modeling was completed in the presence of the appropriate levels of serum E2 for each serum type (male: 0.1 nM, female in follicular phase 0.3 nM, pregnancy 55 nM E2). The observed range of genistein and bisphenol A concentrations *in vivo* in humans are 1–1000 nM and 0.88–200 nM, respectively.[52,71]

for genistein and bisphenol A are 0.32 and 0.002 (Table VII). Because the comparisons for these three compounds involve changes in multiple parameters (i.e., affinities for receptor and binding proteins and clearances), the model was used to illustrate the impact of clearance alone. Keeping the same affinities used for the weak agonist bisphenol A, clearance was varied 10-fold lower and higher resulting in 10-fold differences in dose rates required to obtain 50% receptor occupancy. This contrasts with the predictions

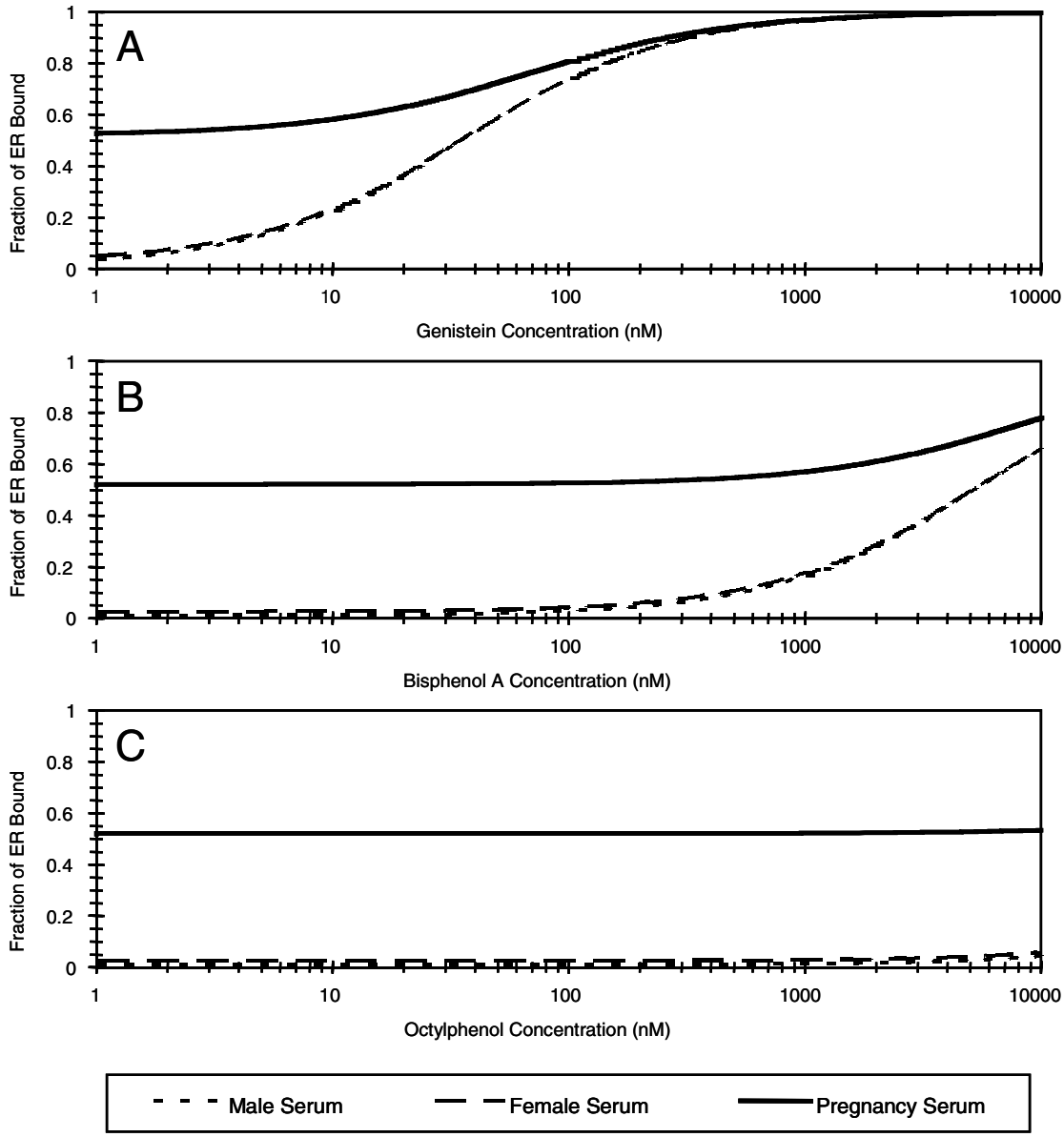

Fig. 8. Modeled total cellular ER binding by E2 and exogenous endocrine active compounds (genistein, bisphenol A, octylphenol) in the presence of human male, female, and pregnancy serum. Modeling was completed in the presence of the appropriate levels of serum E2 for each serum type. E2 concentrations were 0.1 nM for males, 0.3 nM for nonpregnant females in follicular phase, and 55 nM for pregnant females. The observed range of genistein and bisphenol A concentrations *in vivo* in humans are 1–1000 nM and 0.88–200 nM, respectively.[52,71]

of the *in vitro* model for a constant relative activity (Table VII).

4. DISCUSSION

The analyses presented here have extended previous work describing the binding of endogenous hormones to albumin and SHBG in adult human serum.[30,65,66] The previous studies demonstrated the importance of binding for determining the free concentration and free fraction of hormone. They also showed that multiple hormones interacted with the same binding proteins (e.g., SHBG or corticosteroid-binding protein) with two or three competing to a sufficient degree to result in small increases in the free concentration over the predictions for a single hormone. The current work evaluated binding in rat serum, the most commonly used experimental species

Table VI. Modeled EC_{50}s and Relative Potencies for *In Vitro* Assays of Receptor Binding with Rat Serum

	No Serum		Adult Rat Serum		gd19 Fetal Rat Serum		pnd1 Rat Serum		pnd2 Rat Serum	
Assay[a]	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)	EC_{50} (nM)	Free (%)
Estradiol	0.2	100	5.0	4	57	0.4	12	1.7	17	1.2
Genistein	10	100	31	33	18	56	22	46	28	36
Bisphenol A	400	100	5161	7.7	1561	26	3031	13	4391	9.1
Octylphenol	285	100	148,851	0.2	32,386	0.9	81,986	0.4	123,851	0.2

	Potency Relative to E2[b]				
Assay	No Serum	Adult Rat Serum	gd 19 Fetal Rat Serum	pnd 1 Rat Serum	pnd 2 Rat Serum
Estradiol	1	1	1	1	1
Genistein	0.02	0.16	3.17	0.55	0.61
Bisphenol A	0.0005	0.0009	0.037	0.004	0.004
Octylphenol	0.0007	0.000034	0.0018	0.00015	0.00014

[a]Modeled in the absence of endogenous E2, but the results vary by less than 5% in all cases if the endogenous concentrations of E2 reported by Montano *et al.*[26] are modeled as present.
[b]$EC_{50}E2/EC_{50}EAC$.
Note: gd: gestational day, pnd: postnatal day.

for toxicology and endocrinology studies. The interactions with binding proteins of several natural and synthetic compounds were also considered to evaluate the extent of their binding and the potential that they would alter the availability of endogenous hormones. In addition, this effort evaluated literature for fetal and early postnatal periods because these periods are considered critical windows for potential permanent organizational impacts of endocrine active compounds on endocrine function. Finally, a simplified *in vivo* rat model that integrates clearance, plasma-protein binding, and uterine-receptor binding was developed to demonstrate the importance of considering all these factors in estimating the relative potency of endocrine active compounds.

Predictions of E2-free fractions in adult nonpregnant serum from male and female rats and humans gave good consistency with reported experimental findings. Limited experimental data were identified on the distribution of E2 in maternal serum during pregnancy, though total concentrations in humans are very high (i.e., 55 nM vs. <1 nM during menstrual cycling). Binding to elevated SHBG in maternal blood clearly substantially elevates the total concentration and reduces the free fraction of E2, though the modeling here and by Dunn and co-workers[27,30] estimate two- to four-fold lower free fractions than reported by Hammond *et al.*[27] It should be noted that the free concentration of E2 is much higher during pregnancy as compared to males or nonpregnant females (Fig. 7). While there is a substantial elevation in SHBG, it is not enough to prevent the free concentration from rising.

Perhaps the most striking discrepancies in modeled and observed free fractions were for fetal and early postnatal serum in rats and humans. Prediction of measured free fractions of E2 in rat serum required the assumption that the concentration of

Table VII. Comparisons of Relative Potency Predicted Using *In Vitro* and *In Vivo* Models

	Potency Relative to E2		
	In Vitro		*In Vivo*
Compound	Serum Free	Adult Rat Serum	Adult Rat
Estradiol	1	1	1
Genistein	0.02	0.16	0.32
Bisphenol A	0.0005	0.0009	0.002
Weak agonist—slow clearance	0.0005	0.0009	0.02
Weak agonist—fast clearance	0.0005	0.0009	0.0002

αFP available for binding was significantly reduced. This decrease in available αFP likely arises from competition by endogenous fatty acids for which αFP has a major physiological transport role in rodents and humans.[26,67,68] Fatty acids have been demonstrated experimentally to compete for E2 binding to αFP.[68] Predictions of human cord blood concentrations also did not appear consistent with data on free plus albumin-bound hormone. Competition is a factor for drugs binding to albumin and competing with fatty acids and perhaps other compounds during pregnancy.[31,32] Unlike the rodent αFP, no indications were found for fatty acid binding to SHBG.[69,70] However, a variety of phytoestrogens bind to SHBG, so these also may be unaccounted for competitors.[39] This is obviously an area that needs clarification because there have been attempts to estimate relative potencies (i.e., free fractions) in fetal serum based upon data from adult serum.[15] Any such efforts have to be considered speculative, given that it appears the free concentrations may be impacted by factors other than the concentrations and binding affinities of the serum-binding proteins and endocrine active compounds. Furthermore, the dramatically different levels of E2 in perinatal rodent and human serum indicates that thoughtful analysis of appropriate interspecies extrapolation are needed to use results of rodent studies for evaluating potential human health impacts during development.

The impacts of serum-binding proteins on *in vitro* assays must be considered in the overall physiological context in order to evaluate their significance to *in vivo* situations. We demonstrated through simulation that receptor binding measured *in vitro* in the absence of the appropriate composition and concentration of plasma proteins present *in vivo* may overpredict (e.g., octylphenol, 15-fold) or underpredict (e.g., genistein, three-fold) relative potency (Table VI). Moreover, well-designed *in vitro* assays generally do not include metabolism or other forms of clearance (or degradation) of the endocrine active compounds being studied. Thus, the contributions of metabolism and other pharmacokinetic factors must also be considered to develop improved methods for extrapolating *in vitro* potencies to *in vivo*.

A simplified *in vivo* rat model that integrates clearance, plasma-protein binding, tissue partitioning, and uterine-receptor binding was developed to demonstrate the necessity of considering all these factors in estimating the relative potency of endocrine active compounds. Relative to measures of receptor-binding activity *in vitro* in the presence of adult rat serum, the two-fold lower first-order clearance of genistein and bisphenol A compared to E2, increased the relative potency of these two compounds by a similar extent. As a first approximation, the difference in relative receptor-binding activity as measured *in vitro* in the absence of metabolism and *in vivo* in the presence of metabolism will be in rough proportion of the ratio of the clearance rates for the tested compounds. This is illustrated by the comparisons in Table VII of theoretical compounds with identical binding properties, but 10-fold different clearance as compared to bisphenol A, a weak agonist. A slowly cleared compound with the same binding affinities is comparatively more potent *in vivo*, but not *in vitro*. It can reasonably be expected that the difference in the potency of slowly cleared endocrine active compounds such as *o,p*-DDE estimated using *in vitro* assays and *in vivo* assays would reflect these kinds of differences.

We have used the data that is commonly available, ER and plasma-protein-binding affinity, partition coefficients, and estimates of clearance, to demonstrate the impact these processes have on estimated relative potency under some simplifying conditions. The model does not account for homeostatic mechanisms that control levels of steroid hormones. It is possible that administration of a compound that affects change on ER-dependent processes may also result in compensating changes in circulating levels of estrogen, attenuating the potency of the compound, though the extent of such feedback during developmental periods may be more limited than in the adult.

The release of endogenous-bound E2 through competition for binding plasma-protein-binding sites has been raised as a possible mechanism of action of endocrine active xenobiotics.[6] The simulations for human males, females, and pregnant females indicate that genistein, bisphenol A, and octylphenol are not expected to displace biologically significant amounts of E2 from plasma proteins across large concentration ranges that are inclusive of those observed *in vivo* for genistein and BPA.[52,71] For other compounds, the suggestion that release of plasma-protein-bound E2 by competition would lead to increases in free E2 should be treated with caution. Similar concerns about potential release of protein-bound pharmaceuticals have been raised and are generally now discounted.[72,73] For instance, as has been shown for some pharmaceuticals, release of a plasma-protein-bound compound whose clearance is protein binding limited, may increase the free fraction, but will also increase the

fraction available for clearance,[74,75] resulting in compensating decreases in total plasma concentrations.[76] It has been shown that binding of bisphenol A to SHBG administered i.v. to mice reduces the clearance, as would be anticipated with increasing concentrations of a high affinity serum-binding protein.[77]

These analyses have identified important data gaps for implementing quantitative approaches. Notable is a lack of measurements of high capacity, low affinity albumin binding, despite its major role in serum-protein binding, in contrast to extensive efforts to measure low capacity, high affinity binding to SHBG or αFP. Consistent evaluation of binding to the ER from rats and mice, as well as humans, is also essential for good interspecies extrapolations. Much current literature does not allow evaluation of whether differences in ER affinity are species differences or a consequence of a wide variety of experimental methods. We have developed quantitative in *vitro* and *in vivo* models for integrating processes that affect apparent relative potency of endocrine active compounds including binding affinity for the ER and serum-binding proteins and clearance. Together, the approaches developed here provide a useful framework for utilizing experimental data from *in vitro* and *in vivo* studies to estimate the relative potencies of these compounds. Perhaps most importantly, this analysis and others demonstrate that pharmacokinetic and pharmacodynamic considerations not captured in *in vitro* experiments or models can lead to misleading assumptions regarding the potency of endocrine active compounds relative to E2.

ACKNOWLEDGMENTS

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APPENDIX

Equations governing the equilibrium distribution of E2 and genistein in the free and protein-bound forms. Free concentration of E2 in the media:

$$\begin{aligned}\mathrm{E2M} = (&\mathrm{DOSEE2}/(\mathrm{VM}*(1+\mathrm{BPTOT1}/\\ &(\mathrm{K3}*\mathrm{VM}*(1+\mathrm{E2MD}/\mathrm{K3}+\mathrm{GMD}/\mathrm{K4}))\\ &+\mathrm{BPTOT2}/(\mathrm{VM}*(1+\mathrm{E2MD}/\mathrm{K5}\\ &+\mathrm{GMD}/\mathrm{K6}))/\mathrm{K5})+\mathrm{VT}*\mathrm{PE2}\\ &*(1+\mathrm{ERTOT}/(\mathrm{K1}*\mathrm{VT}*(1+\mathrm{E2MD}\\ &*\mathrm{PE2}/\mathrm{K1}+\mathrm{GMD}*\mathrm{PG}/\mathrm{K2}))))),\\ \mathrm{E2MD} = \mathrm{E2M},&\end{aligned} \tag{A1}$$

where DOSEE2 is the amount of E2 added to the system, VM is the volume of the media compartment, and VT is the volume of the tissue compartment. BPTOT1 and BPTOT2 are the amounts of albumin and SHBG or αFP added to the system, respectively. E2M and GM are the concentrations of E2 and genistein in the media compartment; E2D and GMD are the concentrations of E2 and genistein from the previous iteration. K1–6 are the KDs for each ligand:receptor complex: K1 = E2:ER; K2 = genistein:ER; K3 = E2:albumin; K4 = genistein:albumin; K5 = E2:SHBG or E2:αFP; K6 = genistein:SHBG or αFP. PE2 and PG are the media:tissue partition coefficients. All amounts are in nanomoles, volumes in liters, and KDs in nanomoles. Free concentration of genistein (or alternative ligand) in the media:

$$\begin{aligned}\mathrm{GM} = (&\mathrm{DOSEG}/(\mathrm{VM}*(1+\mathrm{BPTOT1}/(\mathrm{K4}\\ &*\mathrm{VM}*(1+\mathrm{E2MD}/\mathrm{K3}+\mathrm{GMD}/\mathrm{K4}))\\ &+\mathrm{BPTOT2}/(\mathrm{VM}*(1+\mathrm{E2MD}/\mathrm{K5}\\ &+\mathrm{GMD}/\mathrm{K6}))/\mathrm{K6})+\mathrm{VT}*\mathrm{PG}\\ &*(1+\mathrm{ERTOT}/(\mathrm{K2}*\mathrm{VT}*(1+\mathrm{E2MD}\\ &*\mathrm{PE2}/\mathrm{K1}+\mathrm{GMD}*\mathrm{PG}/\mathrm{K2}))))),\\ \mathrm{GMD} = \mathrm{GM}.&\end{aligned} \tag{A2}$$

Free concentrations of binding proteins in the media:

$$\mathrm{BP1} = \mathrm{BPTOT1}/(\mathrm{VM}*(1+\mathrm{E2M}/\mathrm{K3}+\mathrm{GM}/\mathrm{K4})), \tag{A3}$$

$$\mathrm{BP2} = \mathrm{BPTOT2}/(\mathrm{VM}*(1+\mathrm{E2M}/\mathrm{K5}+\mathrm{GM}/\mathrm{K6})). \tag{A4}$$

BP1 and BP2 are the free concentrations of albumin and SHBG or αFP, respectively.

Concentrations of bound E2 and genistein in the media:

$$\mathrm{GBP1} = \mathrm{GM} * \mathrm{BP1}/\mathrm{K4}, \quad \text{(A5)}$$

$$\mathrm{GBP2} = \mathrm{GM} * \mathrm{BP2}/\mathrm{K6}, \quad \text{(A6)}$$

$$\mathrm{E2BP1} = \mathrm{E2M} * \mathrm{BP1}/\mathrm{K3}, \quad \text{(A7)}$$

$$\mathrm{E2BP2} = \mathrm{E2M} * \mathrm{BP2}/\mathrm{K5}, \quad \text{(A8)}$$

where GBP1 and GBP2 are the concentrations of genistein-bound albumin and SHBG or αFP, respectively.

Concentrations of free ER, free E2, and ER-bound E2 in the tissue:

$$\mathrm{ER} = \mathrm{ERTOT}/(\mathrm{VT} * (1 + \mathrm{E2M} * \mathrm{PE2}/\mathrm{K1} + \mathrm{GM} * \mathrm{PG}/\mathrm{K2})), \quad \text{(A9)}$$

$$\mathrm{E2T} = \mathrm{E2M} * \mathrm{PE2}, \quad \text{(A10)}$$

$$\mathrm{E2ER} = \mathrm{E2T} * \mathrm{ER}/\mathrm{K1}, \quad \text{(A11)}$$

where ERTOT is the total amount of ER, E2T is the concentration of free E2, and E2ER is the concentration of E2-bound ER in the tissue compartment.

Concentrations of free genistein and ER-bound genistein in the tissue:

$$\mathrm{GT} = \mathrm{GM} * \mathrm{PG}, \quad \text{(A12)}$$

$$\mathrm{GER} = \mathrm{GT} * \mathrm{ER}/\mathrm{K2}, \quad \text{(A13)}$$

where GT is the concentration of genistein and GER is the concentration of genistein-bound ER in the tissue compartment.

In the two-compartment *in vivo* model, the plasma and response tissue concentrations of E2 are described as:

$$d\mathrm{APE_{E2}}/dt = \mathrm{QRT} * (\mathrm{CVRT_{E2}} - \mathrm{CP_{E2}}) + \mathrm{RADOSE_{E2}} - \mathrm{KELIM_{E2}} * \mathrm{APT_{E2}}, \quad \text{(A14)}$$

$$d\mathrm{ART_{E2}}/dt = \mathrm{QRT} * (\mathrm{CP_{E2}} - \mathrm{CVRT_{E2}}), \quad \text{(A15)}$$

where $\mathrm{AP_{E2}}$ is the amount of E2 in the plasma, $\mathrm{ART_{E2}}$ is the amount of E2 in the response tissue, QRT is the plasma flow to the response tissue, $\mathrm{CVRT_{E2}}$ is the venous concentration leaving the response tissue and entering the plasma compartment, $\mathrm{CP_{E2}}$ is the free E2 concentration calculated using Equation (A1), $\mathrm{RADOSE_{E2}}$ is the constant infusion dose rate, $\mathrm{KELIM_{E2}}$ is the first-order elimination rate constant calculated in the methods, and $\mathrm{APT_{E2}}$ is the total concentration of E2 in plasma. The response tissue has ER, so Equations (A9)–(A11) are used to calculate receptor occupancy. Equations for genistein and other compounds are identical, substituting appropriate subscripts.

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